Chapter 8
Enchytraeid reproduction test

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According to international regulations (e.g., from the European Union [EU]), the environment should be protected against hazardous effects of chemicals. Following accepted rules, the ecotoxicological risk of these substances must be assessed based on test data. For the terrestrial environment, this assessment depends mainly on acute and long-term earthworm tests (*Eisenia fetida, E. andrei*) according to the Organization for Economic Cooperation and Development (OECD) [1] or Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA) [2] guidelines. The 2 *Eisenia* species were selected as test organisms mainly because they are easy to breed and have, in comparison to other Lumbricidae, a short life cycle. Unfortunately, these earthworm species normally do not occur in soils but in organic matter such as compost. Therefore, for risk assessment purposes it is not fully scientifically justified to regard *E. fetida* and *E. andrei* as typical representatives of the soil community [3].

Additionally, parallel to testing strategies for the aquatic medium, there is a growing need for further tests with soil organisms [4]. Species from different trophic levels and different taxonomic, physiological, and/or functional groups should be selected and long-term endpoints should be measured in order to improve the risk assessment of chemicals in soil [5]. In such a tiered test strategy with increasing ecological relevance and complexity, a test battery with, e.g., Collembola, Carabidae, Staphylinidae, or Enchytraeidae (Potworms; Oligochaeta), could be used.

In this chapter, the new Enchytraeid Reproduction Test (ERT), with the potworm, *Enchytraeus albidus*, is presented. Recently, a draft protocol of this test was used as a basis for an international validation study [6]. The most important part of this validation process is the performance of a ring test that started in autumn 1996. Some general aspects of ring test performances will also be presented. Before describing the test and some preliminary results in detail, information on the ecology of these small and inconspicuous animals and their use in ecotoxicology is given. The following issues will be discussed:

1) biology of terrestrial Enchytraeidae,
2) history of various test approaches using enchytraeids,
3) description of the ERT,
4) presentation of selected test results,
5) comparison of the ERT with existing earthworm tests, and
6) status of the international ring test.

Biology of terrestrial Enchytraeidae

Enchytraeids have been known worldwide since the second half of the 19th century. However, the ecological role of these small (usually 1 to 20 mm), inconspicuous, and often whitish worms has hardly been studied. This lack of knowledge is at least partly due to the difficult taxonomy of the family in general. Altogether, several hundred species have been described from terrestrial habitats, but only a relatively small number of species (usually 5 to 25) occur at any given field site. The increasing number of soil biological studies as part of various acid rain projects during the 1970s and 1980s contributed significantly to the understanding of the role of Enchytraeids in the soil ecosystem.

Enchytraeids are important members of the soil biocenosis (at least in temperate regions) in many different habitats, especially where earthworms are rare [7]. At sites with low pH and a high content of organic matter, they can reach annual average densities of more than 100,000 individuals per m^2 (Table 8-1). Such high densities, e.g., in coniferous forests, are usually caused by few species, whereas the majority of the Enchytraeids prefer slightly acidic to alkaline soils. At many sites (including mull soils), they show compared to the earthworms, the highest biomass of all other invertebrate groups [8]. Additionally, a significant contribution of Enchytraeids to total soil respiration has been found [8]. Despite their small individual size, Enchytraeidae probably influence soil structure evolution by producing excrements, burrowing actively, and transporting of soil particles [7]. Even though some species might act as primary decomposers, other Enchytraeids are known to play a key role affecting the structure or function of the soil microflora [9,10]. Therefore, reduction of these oligochaete populations by chemicals may reduce the decomposition rate even if there is no direct effect on microorganisms.

Enchytraeids prefer to live close to or in soil pore water and consequently are directly exposed to dissolved chemicals. They have species-specific feeding preferences (e.g., fungi, bacteria, algae, protozoa, collembola excrements, and organic material like litter, often in combination with soil particles). Thus, their exposure to chemicals could occur from the surrounding soil water or from the food. From field studies it is known that enchytraeids react sensitively to a wide variety of anthropogenic stressors like heavy metals [11], petroleum [12], or pesticides [13]. However, the reaction of the whole coenosis is often very differentiated, since the various species are differently exposed and affected, depending on their preferred soil depth or food. For example, it seems that species of the genus *Enchytraeus* are more sensitive than species from other genera under field conditions [14].

History of test approaches using enchytraeids

Enchytraeids (especially *Enchytraeus albidus*) have been used in ecotoxicological laboratory tests for more than 30 y (Table 8-2). The first was a late-1960s test with *Enchytraeus albidus* in water to assess the effects of anthelminthics, but it was not until the early 1990s that several groups, working independently from each other, started to develop different test approaches for enchytraeids. In these test approaches, test substrates like water, agar, or soil were used (Table 8-3). However, the behavior of a chemical in agar or

Table 8-1 Abundance, biomass, and number of species of Enchytraeidae in Central Europe [7, 30]

Biotope	Average ± SD [Ind./$m^2 \times 10^3$]	Minimum	Maximum
Moor	45.5 ± 47.4	3	142
Meadow (pH < 5.5)	41.4 ± 20.2	31	74
Meadow (pH > 5.5)	20.6 ± 12.6	5	24
Conif. forest (Mor)	51.0 ± 47.6	10	134
Conif. forest (Raw Humus)	53.5 ± 55.34	4	134
Decid. forest (Mull)	17.3 ± 9.7	6	39
Decid. forest (Mor)	60.9 ± 56.1	5	146
Ruderal places	25.2 ± 21.2	6	56
Arable land	10.5 ± 7.2	2	30
Sewage sludge	≈ 2390	?	?
Biotope	**Average ± S.D [g/m^2]**	**Minimum**	**Maximum**
Moor	0.94 ± 1.00	0.09	2.38
Meadow (pH < 5.5)	0.84 ± 0.51	0.53	1.76
Meadow (pH > 5.5)	0.59 ± 0.27	0.34	0.97
Conif. forest (Mor)	1.13 ± 0.53	0.50	1.94
Conif. forest (Raw Humus)	0.82 ± 1.25	0.17	2.69
Decid. forest (Mull)	0.59 ± 0.47	0.23	1.60
Decid. forest (Mor)	0.95 ± 0.62	0.40	1.64
Ruderal places	0.47 ± 0.39	0.13	0.90
Arable land	0.37 ± 0.21	0.11	0.64
Sewage sludge	≈ 206.8	?	?
Biotope	**Average ± SD [# of species]**	**Minimum**	**Maximum**
Moor	9.1 ± 7.3	1	23
Meadow (pH < 5.5)	8.3 ± 8.8	4	28
Meadow (pH > 5.5)	10.7 ± 8.2	3	24
Conif. forest (Mor)	9.5 ± 6.9	2	19
Conif. forest (Raw Humus)	4.3 ± 0.6	1	14
Decid. forest (Mull)	25.7 ± 7.4	18	36
Decid. forest (Mor)	16.1 ± 9.2	7	27
Ruderal places	8.0 ± 3.9	2	13
Arable land	9.1 ± 7.7	3	22
Sewage sludge	2	1	3

water is not comparable to that in soil, and thus the conditions for a worm are quite different compared to its normal habitat. Therefore, for risk assessment purposes only, soil tests are useful because otherwise the extrapolation to soil quality criteria is rather difficult [3]. It is useful to perform tests simultaneously in different substrates. In tests using agar as a substrate, the measurement endpoint of cocoon production can be used, which would be difficult to determine in soil. From an ecological point of view, assessing of the number of those animals that reproduce is more important than assessing the number

Table 8-2 Historical overview on enchytraeid tests

Year	Reference	Substrate, type, species, chemical
1968	Weuffen [31]	Water; acute; *E. albidus*; diff. Anthelminthika
1975	Kaufman [32]	Soil; acute; *E. albidus*; Phenol
1984	Heungens [33]	Litter; acute; *C. sphagnetorum*; pH, salts
1984	Huhta [34]	Soil, litter; acute; *C. sphagnetorum*; pH, nutrients
1989	Römbke [35]	Soil; prolonged; *E. albidus*; brass
1989	Römbke & Knacker [36]	Water; acute; *E. albidus* + 4 other species; 8 chemicals
1989	Westheide et al. [37]	Agar; prolonged; *E. crypticus*; 9 chemicals, liquid manure
1990	Rüther & Greven [18]	Agar; acute, accumulation; *E. buchholzi*; 4 metals
1991	Funke & Frank [38]	Soil; acute; natural cenosis; diff. volatile hydrocarbons
1991	Graefe [39]	Water; acute; *E. minutus, E. lacteus*; soil extracts
1991	Purschke et al. [40]	Agar; prolonged; *E. crypticus*; diff. pesticides
1991	Römbke [16]	Soil; prolonged; *E. albidus, E. crypticus*; diff. chemicals
1991	Westheide & Bethge-Beilfuß [41]	Agar (+ soil); prolonged; *E. crypticus*; 3 pesticides
1991	Westheide et al. [42]	Agar, soil; prolonged; *E. crypticus, E. minutus*; Benomyl
1992	Mothes-Wagner et al. [43]	Soil (microcosm); prolonged; natural cenosis; chemicals
1993	Born [44]	Soil (microcosm); prolonged; natural cenosis; Aldicarb
1993	Elzer [45]	Soil; prolonged; *E. buchholzi*; lead, cadmium
1994	Dirven-van Breemen et al. [17]	Soil; prolonged; *E. albidus, E. crypticus*; Zinc
1994	Notenboom & Posthuma [46]	Soil; prolonged; *E. albidus, E. crypticus*; 4 metals
1994	Römbke et al. [15]	Soil (microcosm); prolonged; natural cenosis; 2 pesticides
1994	Willuhn et al. [47]	Agar, water; acute; *E. buchholzi*; cadmium
1995	Achazi et al. [48]	Soil, agar, water; prolonged; *E. albidus, E. crypticus*; 3 chemicals + contaminated soils
1995	Christensen et al. [49]	Water; acute; *E. bigeminus*; 3 pesticides
1995	Heck [50]	Soil; prolonged; *E. albidus, E. crypticus*; PAKs + PCBs
1995	Kristufek et al. [51]	Agar; prolonged; *E. crypticus*; 7 chemicals
1995	Römbke & Federschmidt [14]	Soil; prolonged; *E. albidus, Fridericia sp.*; Carbendazim
1995	Rundgren & Augustsson [52]	Soil; prolonged; *C. sphagnetorum*; Cu, LAS, Dimethoate

Table 8-3 Advantages and disadvantages of different test substrates

Approach	Advantages	Disadvantages
Agar	Easy, various reproduction parameters measurable	Exposure not comparable to soil or water
Water	Easy, quick, inherent toxicity	Only 1 exposure pathway
Pore water	Same as water	Same as water
Artificial soil	Comparable, standardized test substrate	Different from natural soils e.g., organic content too high
Natural soil	Ecological reality; regional situations accessible	Highly variable; too many different possibilities
Sediment	Same as soil	Same as soil

of cocoons. Most of the tests listed in Table 8-1 were performed in the context of field validation or monitoring studies. The ERT, however, was developed as a standard test designed for the notification and registration of chemicals.

In the laboratory, a high number of stress factors have been tested that show an effect on enchytraeids, including heavy metals, environmental chemicals, pharmaceuticals, and at least 13 pesticides [e.g., 15,16,17], (Table 8-4). Enchytraeids are also useful for bioaccumulation studies, especially with heavy metals [18; Chapter 10, this volume]. Nearly all test methods using soil as a substrate can be modified for soil quality assessment (the ERT is already listed as a possible method for this purpose [19]). However, in this chapter, only the testing of single substances (e.g., as part of the registration process of pesticides) will be discussed.

Table 8-4 Stress factors tested with enchytraeids in the laboratory

Group	Type
Insecticides	Aldicarb, DBCP, dicofol, dimethoat, lindan, parathion, propoxur
Herbicides	2,4,5-T, amitrole, diuron
Fungicides	Benomyl, carbendazim, pentachlorophenol
Environmental chemicals	Benzothiaxole, chloroacetamide, chloroform, potassium dichromate, tetrachloroethene, trichloroethene, TPBS
Heavy metals	Cadmium, copper, lead, zinc
Pharmaceuticals	Various (» 50) anthelminthic acitive ingredients
Other stress factors	Ash, lime, nitrogen, sulphuric acid, soil cultivation

Despite the fact that many different species were examined, only species of the genus *Enchytraeus* are widely used in ecotoxicological tests. This is due to the simple culturing and handling of many but not all species of this genus. Species like *E. albidus, E. buchholzi, E. coronatus*, or *E. crypticus*, which can reach enormous densities in laboratory cultures, can be well fed with rolled oats [20]. All other enchytraeid species, probably with the exception of *Cognettia sphagnetorum* [21], are too difficult to breed in mass cultures, and therefore cannot be used as standard test organisms.

Within the genus *Enchytraeus*, various species have been proposed for testing purposes. Test results are available for *E. albidus* and for some species of the *E. minutus/E. buchholzi* complex. *E. albidus* has been promoted mainly because of its size (adult animals have a length of approximately 10 to 15 mm) (Figures 8-1 and 8-2) and the simple breeding method of this species, which has been kept in culture as fish food for decades [22]. Difficulties with the handling of small species like *E. crypticus* can be avoided when staining is applied instead of wet extraction for the purpose of counting juveniles. Still there is the question of ecological relevance: *E. albidus* is distributed worldwide in various biotopes, whereas the origin of *E. crypticus* is not known (the species was described from a compost heap). However, other recently extracted species of the *E. minutus* complex are abundant in German meadow soils and can be bred easily, too. In fact, including various species in the annex of the ERT guideline is planned, since the test performance is not affected by the choice of species. The same procedure was used in other recently finalized Organiza-

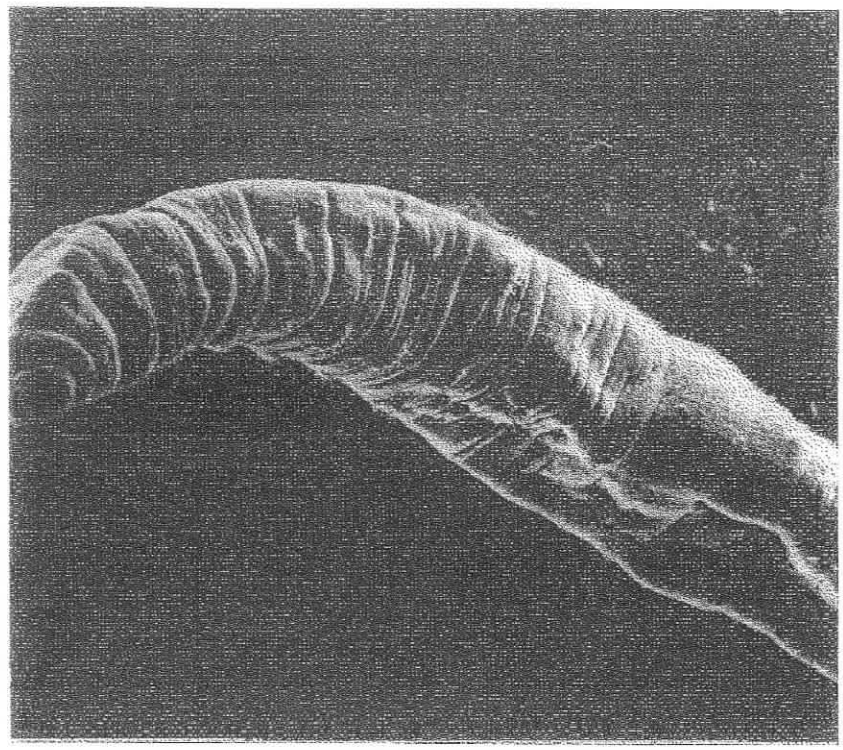

Figure 8-1 Scanning electron microscope picture of the anterior body of *Enchytraeus albidus* (Enchytraeidae, Oligochaeta); magnification factor: 25. The characteristic bundles with 4 setae are clearly visible.

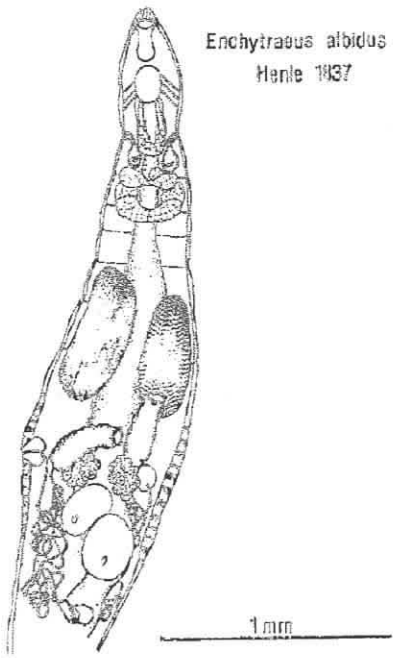

Figure 8-2 Drawing of internal organs of an adult *Enchytraeus albidus* (Enchytraeidae, Oligochaeta). Note the extraordinary long and coiled sperm duct in the lower half of the body cavity behind the clitellum.

tion for Economic Cooperation and Development (OECD) guidelines (e.g., the new guideline on fish bioaccumulation, which is currently discussed as a final draft).

Materials and methods

Description of the Enchytraeid reproduction test

The actual version of the ERT protocol, used in the recently finished international ring test, is described briefly. Further details of the test development and results of tests with various plant protection products and environmental chemicals (partly with slightly different versions) can be found in [16] or [17]. Listed below are the criteria details of the protocol:

- Guideline: Draft guideline according to OECD standards
- Test species: *Enchytraeus albidus*; *Enchytraeus sp.*; 10 adult worms (as identified by visible eggs in the clitellum region)
- Substrate: Artificial soil according to OECD [1]
- Parameter: Range-finding test: mortality, behavior
  Reproduction test: number of juveniles
- Performance: 4 replicates; room temperature (20 ± 2 °C); weekly feeding oats strewn onto the soil surface); removal of the adult worms after 3 weeks; counting of the juveniles hatched after 3 more weeks after extraction by a wet funnel method (however, staining with Bengal red seems to be more efficient and time-saving, as proposed by W. de Coen, University of Ghent, Belgium, and used, e.g., by RIVM Bilthoven [17])
- Concentration: Range-finding test: 0.1, 1, 10, 100, 1000 mg/kg
- Reproduction Test: No-observed-effects concentration (NOEC) approach: at least 5 concentrations (4 replicates)

  EC_x-approach: at least 12 concentrations (2 replicates)
- Reference substance: Carbendazim (formulation Derosal)
- Validity: In the control: after 3 weeks mortality < 10%; after 6 weeks number of juveniles > 25 per vessel
- Evaluation: LC50: Probit analysis; NOEC estimation: analysis of variance (ANOVA)

  EC_x: Regression analysis or similar methods

Results

Presentation of selected test results

Two tests were performed at ECT GmbH according to the ERT draft guideline in autumn of 1996. The fungicide benomyl (a.i. benomyl: 50%) was selected because it is metabolized quickly in soil to the proposed reference chemical carbendazim. There was nearly no mortality observed in the NOEC approach (mean survival rate: 97.3 ± 3.0%) as well as in the EC_x approach (97.6 ± 3.8%). Due to the low number of dead worms, no statistical evaluation was possible. The measured NOEC for reproduction is 4.04 mg/kg (multiple sequentially U-test after Bonferroni). For the EC_x approach, a dose-response relationship is not recognizable (Figure 8-3).

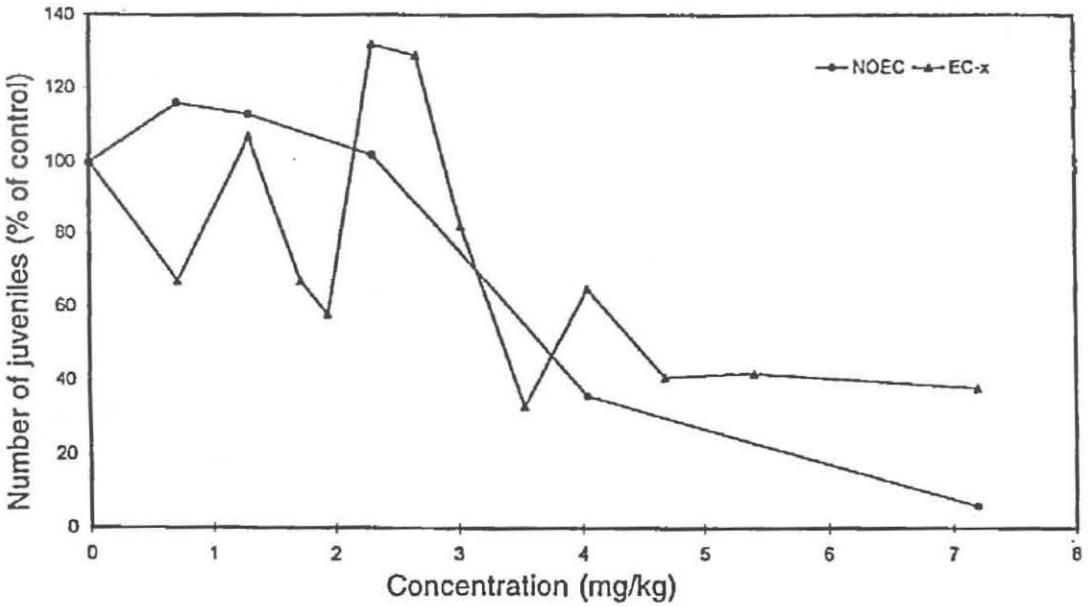

Figure 8-3 Effects of benomyl (a.i. benomyl: 50%) on the reproduction of *E. albidus* (ERT: Comparison of NOEC and EC_x approach). All data are given in mg/kg dry weight artificial soil.

In the second test, the fungicide Derosal (a.i. carbendazim: 36%) was used. The results of the 2 approaches show reasonable consistency. According to the validity criteria of the ERT draft guidelines, the test can be considered valid. The NOEC of the mortality data is 5.6 mg/kg (Williams-Test). The mean survival rate in the control and in the 4 lowest concentrations of the NOEC approach is 96.2 ± 2.5%; in the LOEC (10.0 mg/kg), the survival rate is 87.0 ± 0.5%. EC_x determination was impossible because a dose-response relationship was not observed and the average mortality was low (mean survival rate: 97.4 ± 3.8%). To calculate the NOEC of the reproduction data, the second concentration was excluded because of the increase in the number of juveniles (Figure 8-4). The determined NOEC for reproduction is 3.2 mg/kg (ANOVA). In a first statistical approach used to evaluate the EC_x, all concentrations producing more juveniles than the control had to be

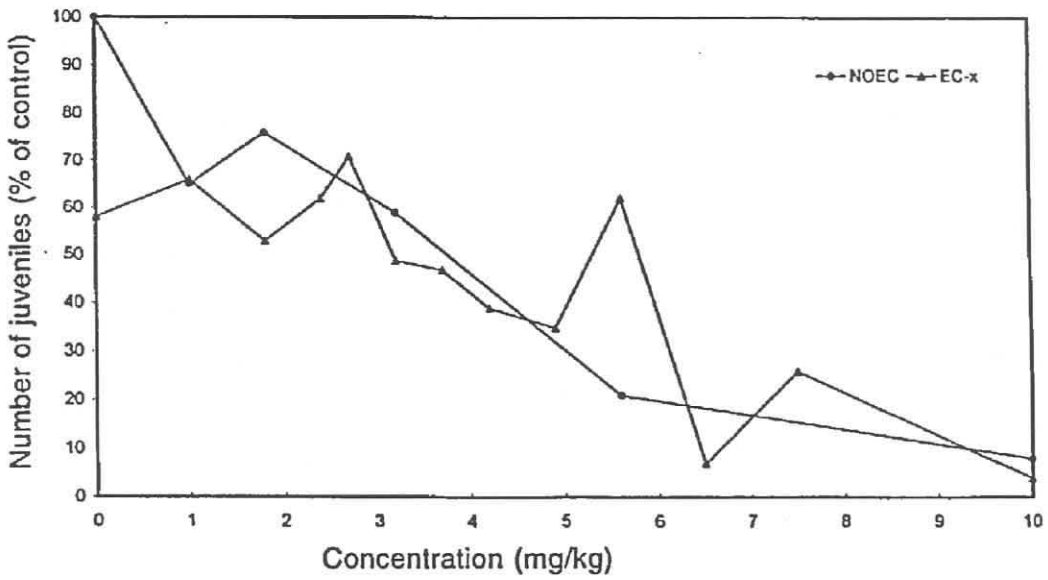

Figure 8-4 Effects of Derosal (a.i. carbendazim: 36%) on reproduction of *E. albidus* (ERT: Comparison of NOEC and EC_x approaches). All data are given in mg/kg dry weight artificial soil.

excluded. An increase in the measured endpoint (# of juveniles) at low concentrations, i.e., a hormesis effect, was originally described from plant tests but seems to be very common in many different botanical and zoological test systems. Therefore, only 8 concentrations were used for the determination of the EC10: 2.5 mg/kg (confidence limits [95%]: 1.0 to 3.4 mg/kg).

The test results for Derosal obtained so far by other participants are within the same order of magnitude. The average mortality of adult worms was low (mean survival rate: 90.7 ± 6.9%). Additionally, the mortality in the tested range of sublethal concentrations (1.0 to 10.0 mg/kg Derosal) was low (LC50 > 10.0 mg/kg). The NOEC and EC10 values varied between 1.0 and 6.3 mg/kg.

These results are within the range that could be expected from data in the literature (e.g., [14]). For example, LC50 values for enchytraeids in laboratory tests can vary between approximately 20 and 30 mg/kg and NOEC values between 1 and 10 mg/kg Derosal, depending on the test species and soil type. LC50 or NOEC values for Derosal from acute and long-term tests with earthworms according to OECD or BBA guidelines are in a similar order of magnitude when recalculated to active ingredient concentrations [23].

Discussion

Comparison of the ERT with existing earthworm tests

In order to assess the applicability of the existing earthworm test protocols and the ERT draft guideline, their pros and cons should be discussed. Concerning the sensitivity of the test species toward chemicals, there is no clear answer. It can be assumed that for each

chemical a different species is the most sensitive. For example, some mites are extremely affected by Cd, whereas earthworms seem to be very sensitive to Cu [24]. After completion of the ring test, a detailed comparison of Enchytraeid and earthworm sensitivity toward chemicals will be presented, which will show not only single test results for a specific chemical but also ranges of sensitivity for some chemicals.

The decision process for 1 of the 2 test methods using *Enchytraeus sp.* or *E. fetida* and *E. andrei* should be based on more than just sensitivity, and these criteria might include the following aspects:

Ecological relevance: Some soil-inhabiting species of the genus *Enchytraeus* (mainly some small species, but also *E. albidus)* are ecologically more relevant than *E. andrei* and *E. fetida*, since the latter occur almost exclusively in compost heaps [3]. A soil-inhabiting lumbricid species like *Aporrectodea caliginosa* would be, with regard to ecological relevance, a better choice of species for the earthworm test. However, tests performed with true soil-living earthworm species revealed breeding problems and long generation cycles [25]. It is not clear whether a fragmenting (i.e., genetically uniform) *Enchytraeus* species could be useful for test purposes.

Practicability: Many *Enchytraeus* species are very easy to handle and to breed. Moreover, their generation time is significantly shorter than for earthworms (test duration depending on the species: 4 to 6 weeks compared to 12 weeks). Due to the small amount of soil necessary for the test (20 g in 1 test vessel, compared to 500 g), only small amounts of contaminated soil are produced in the laboratory. Overall, the enchytraeid test performance is less expensive because of the short test period, the minimum of space required, and except for the start of the test, the small amount of work.

Data availability: Since the earthworm test guidelines were established in 1984 (acute) and 1994 (long-term or chronic), much experience and data have become available. Based on this experience and despite the fact that only a limited number of studies have been performed with enchytraeids so far, the standardization according to OECD rules for the enchytraeid reproduction test has reached a high level in a short period of time.

Extrapolation to semi-field and field conditions: The comparison of laboratory test results with semi-field and field conditions by performing long-term microcosm and field studies within tiered risk assessment schemes [15] can be achieved much easier with Enchytraeids than with earthworms because the same or very similar (same genus) species can be used at all tiers. For earthworms, at the moment, the species recommended for the acute test (Tier 1) and the long-term (reproduction) test (Tier 2) belong to different genera from different ecological groups (epigees, endogees, and aneciques). To solve this problem, an earthworm reproduction test is listed as a possibility for Tier 1 testing in a recent proposal for an ecotoxicological test strategy [5]. In the same proposal, the ERT is mentioned as an option (i.e., as part of a battery of standardized tests that are

required only in special situations) for Tier 2. Further experience from the ongoing ring test and a recently started microcosm test should show whether the obvious advantages of the ERT in a tiered test approach in combination with a tiered risk-assessment scheme are important enough to eventually replace the existing earthworm laboratory tests.

Status of the international ring test

The aim of the ring test is to provide data to assess the suitability of the ERT draft guideline for the risk assessment of chemicals. Concerning legal requirements, the ERT is already included in the appendix of the European Union Technical Guidance Document 93/67/EEC + 1488/94 for the notification of existing and new environmental chemicals. In general, the development and validation of a new test guideline can be classified in the following steps [26] (the schedule for ERT is shown in parentheses):

1) test development (1988 to 1991);
2) prevalidation (informal studies in several labs; 1991 to 1995);
3) validation (currently performed and sponsored by German Federal Environment Agency [UBA] and European Chemical Bureau (ECB);
4) independent assessment of study results and protocols (planned for 1998); and
5) progression toward regulatory acceptance, e.g., finalization as an OECD guideline (planned for 1998).

About 25 institutions have agreed to participate in the ring test. Approximately 10 other institutions are interested in conducting at least some test runs or would like to contribute otherwise to the success of the ring test. Universities, governmental institutions, industry, and contract research laboratories joined the group. Geographically, Germany is represented very well, but nearly all other nations of the EU are also participating. Additionally, laboratories from the Czech Republic, Russia, Canada, the United States, Japan, and South Africa are interested in the ring test.

For the practical work, the fungicide carbendazim (formulation Derosal) and the industrial chemical 4-nitrophenol were selected. Some participants also tested potassium chloride (KCl), which was recently proposed as a reference chemical for earthworm tests [27]. The actual ring test is performed using 2 different statistical approaches, the NOEC- and the EC_x-test design (5 concentrations × 4 replicates and 12 concentrations × 2 replicates, respectively). For the NOEC approach, 28 test vessels are required, while for the EC_x approach, 30 test vessels are used; the number of control vessels is 8 and 6, respectively. The background for this laborious way of performing the ring test is related to the recent discussion about whether the well-known NOEC or a somehow defined and calculated EC_x (e.g., EC10, EC25) value is better suited for risk assessment purposes [25,28].

Based on the experiences gained in the ring test, the ERT draft guideline has been already improved, in particular by reduction in the amount of food in order to avoid molding by fungi and use of staining methods (e.g., Bengal red) to facilitate the counting of juveniles.

However, further improvements are necessary in order to reduce the variability of the test results. Besides the work aimed at upgrading the draft guidelines and completing the

tests with the second test chemical, some participants agreed to do further investigations. For example, other species of the genus *Enchytraeus* and other test substrates, especially natural soils (e.g., LUFA standard soil 2.2) will be tested in order to improve the extrapolation of test data from laboratory to field conditions. Finally, all experience gained in this validation process will be published in a way comparable to those data from other recent ecotoxicological ring tests, e.g., with fish [29].

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